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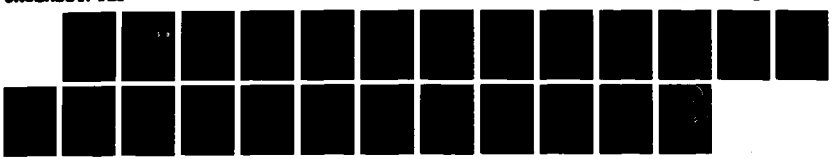

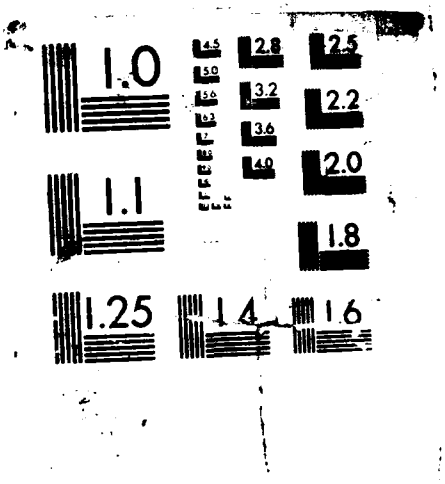

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STUDY OF THE PATHOGENICITY OF AEROMONAS HYDROPHILA FOR MAN

FINAL REPORT

HERBERT L. DUPONT, M.D.

JULY, 1986

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Five Aeromonas hydrophila strains were selected for study from among more than 50 candidate strains collected from various countries. They were selected for the study because of possession of well characterized virulence properties or because they were clearly implicated in cases of human diarrheal illness. Each strain was fed to groups of 3 or 4 adult volunteers. The strains were shed by the volunteers only sporadically and a sustained illness did not develop. This study failed to show a relationship between virulence properties as we now understand them and pathogenicity of Aeromonas for human subjects. Two non-enterotoxigenic enteroadherent E. coli (EAEC) strains not belonging to EPEC serogroup isolated from U.S. students with diarrhea acquired in Mexico were selected for study in volunteers. In doses of 7×10^8 and 1×10^{10} viable cells, strain JM 221 produced a diarrheal illness in 5 of 16 subjects while 1 of 8 fed strain 189 developed a diarrheal illness. The test strains were recovered from stool throughout the study indicating intraluminal or intraintestinal replication of the test strains. The Study confirmed the human pathogenicity of EAEC.

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Summary

Five Aeromonas hydrophila strains were selected for study from among more than 50 candidate strains collected from Thailand, Western Australia, Canada, and the United States. They were selected for the study because of possession of well characterized virulence properties or because they were clearly implicated in cases of human diarrheal illness. All were felt to be pathogenic by laboratory assays. The strains were tested biochemically and toxin production and hemagglutination patterns were characterized. Each strain was then fed to groups of 3 or 4 adult volunteers. The strains were shed by the volunteers only sporadically and except for two volunteers transcently passing unformed stools, a sustained illness did not develop. This study failed to show a relationship between virulence properties as we now understand them and pathogenicity of Aeromonas for human subjects.

In studies looking at the etiology of diarrhea among groups of U.S. adult students in Mexico we found that non-EPEC serotypes of enteroadherent E. coli (EAEC) were isolated from 12% of diarrhea cases overall and from 30% of the patients from whom no other agents were identified. Six of 13 students from whom paired sera were collected showed a fourfold or greater rise in antibodies in serum. Two stains of EAEC were selected for study in volunteers. In doses of 7×10^8 and 1×10^{10} viable cells, strain JM 221 produced a diarrheal illness in 5 of 16 subjects while 1 of 8 fed strain 189 developed a diarrheal illness. The test strains were recovered from stool throughout the study indicating intraluminal or intraintestinal replication of the test strains.

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Foreword

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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Nature of Work Being Reported

The annual report deals with 24 of a total 24 months months concerning contract No. C-3024. The project has dealt with the establishment of a volunteer pool, the accumulation and evaluation of test strains and the administration of several dosages of 5 strains of A. hydrophila and 2 strains of enteroadherent Escherichia coli to a total of 81 healthy adult volunteers.

Statement of the Problem

Approximately 50% of acute diarrhea is associated with the passage of unformed stools which are negative for all recognized enteric pathogens. Studies carried out in many regions of the world have shown that antimicrobial agents can be used to successfully treat or prevent a portion of the illness offering indirect evidence that as yet undefined bacterial agents will prove to be important causes. Two leading bacterial candidates as potential causes of the undiagnosible illness are A. hydrophila and enteroadherent E. coli (EAEC). A. hydrophila has been isolated in a high frequency of diarrhea cases which occur in Thailand, Western Australia, and Canada. EAEC strains have been epidemiologically incriminated as important enteropathogens among U.S. students traveling to Mexico and may explain up to one-third of the previously undiagnosable illness. This study was designed to establish the virulence of these strains for man and to characterize the pathogenesis of infection for those capable of producing illness. Additionally, this volunteer program remains one of the few facilities in the country where enteric infections can be induced in human subjects for the purpose of studying experimental infection.

Background

Diarrheal diseases are a major cause of morbidity in all areas of the world and mortality in developing regions. A large proportion of acute diarrhea cases have no recognized cause, yet these cases often respond promptly to antimicrobial therapy (1-3). This finding suggests that undefined bacterial agents may be responsible for acute diarrhea.

Several characteristics associated with the virulence in other bacterial enteropathogens have been described among strains of Aeromonas hydrophila. Most strains produce a heat-labile cytotoxin as well as enterotoxins (4-6). Another report suggests that some strains may produce a cholera-like toxin (Houston, ASM, 1984). Other A. hydrophila are also capable of hemagglutinating human erythrocytes (7,8). As with enterotoxigenic E. coli, this property is associated with a fimbriae that are able to mediate adherence to the intestinal mucosa.

A. hydrophila is currently considered a human pathogen and has been for some time (9,10). More recently, some A. hydrophila have been associated with cases of diarrhea in children and adults (4,5,11,12). However, this organism is ubiquitous and is frequently found in the stools of healthy persons (4,6,9,13,14). Although a statistical relationship exists between illness and fecal recovery of A. hydrophila, definite proof of pathogenicity for man is lacking. Oral administration of whole cultures to rhesus monkeys, generally felt to be an animal model closely resembling human infection, failed to cause diarrhea (4). This study was designed to determine if A. hydrophila strains

were capable of causing diarrhea in humans when challenged with whole, viable cultures.

We have conducted a number of studies in Mexico looking at the epidemiology, etiology, therapy and prevention of diarrhea in young adults from the U.S. during short term stay there (1,2,15,16). This setting closely resembles the situation of U.S. military populations during short term relocation in the developing world. We became interested in the observation that antimicrobial agents could successfully treat and prevent the diarrhea even when an agent could not be identified in stool (1,2,17). Through a study of serotype and recognized virulence properties of the E. coli isolated from diarrheal stools we identified an agent heretofore not described to be associated with diarrhea (18,19). Initially, we failed to identify common serogroups among the E. coli isolated from diarrheal stools, and furthermore found that enteropathogenic E. coli (EPEC) serogroups were encountered only rarely. Of greater interest, however, E. coli from illness cases commonly were found to adhere to HEp-2 tissue culture cells, a model of pathogenicity for EPEC strains. We have called these HEp-2 adherent strains enteroadherent E. coli (EAEC) to avoid reference to EPEC serotypes (Figure 1). EAEC were found in 42 of 349 (12%) of the illness specimens and 7 of 121 (6%) of the well controls. When looking at the group of ill students with no recognized enteropathogen in their stools, 26 of 89 (29%) had EAEC strains. Six of the 13 available paired sera showed a fourfold or greater rise in antibody titer to the somatic antigen of homologous strain (19). These studies were offered evidence of a new; unrecognized cause of diarrhea and raise important questions as to current thinking about the relationship of serotype to pathogenicity among E. coli.

Approach to Problem

Aeromonas Studies

Test Strains

Five human isolates of A. hydrophila were selected for virulence assays and volunteer studies (Table 1). All strains were lyophilized upon receipt and stored at room temperature. A new lyophile of the same lot was used for each study.

Biochemical Characterization

All strains were confirmed as A. hydrophila using the API-20E identification system (Analytab Products, Plainview, NY). In addition, strains were tested for the production of DNase using DNase test agar (Difco, Detroit, MI) supplemented with oxgall and crystal violet, as previously described (21).

Antimicrobial Susceptibility Testing

Antibiograms were determined using a standard disc diffusion method (Kirby-Bauer). All strains were tested for susceptibility to ampicillin (AM), tetracycline (TE), trimethoprim/sulfamethoxazole (STX), gentamicin (GM), furazolidone (FX), doxycycline (D), and sulfisoxazole (G). Standard ATCC strains of E. coli, Staphylococcus aureus, Streptococcus fecalis and Pseudomonas aeruginosa were used as controls.

Table 1. Source of Strains of
Aeromonas hydrophila

Strain	Location	Site of Infection
6Y	Bangkok (Echeverria)	Stool (asymptomatic)
B158	Perth (Gracey)	Wound
3647	Perth (Gracey)	Stool (diarhea)
SSU	U.S.-C.D.C. (C. Houston)	Stool (diarrhea)
3284	Perth (Gracey)	Stool (diarrhea)

Figure 1. An adherent E. coli strain in the HEp-2 assay (right).
A nonadherent E. coli strain in the HEp-2 assay (left).

Toxin Assays

Hemolysin activity was assayed using 5% sheep and rabbit blood agar plates (Remel Media, Houston, TX). After inoculation and overnight incubation, the plates were examined for beta hemolysis.

Y-1 adrenal tissue culture cells (YAC) were used to measure cytotoxic activity. Cell free supernatants, prepared as previously described (21,22), were added in 100 to 1 amounts to confluent YAC monolayers. Following an 18-24 hour incubation, the monolayers were examined for cytotoxic activity (detached cells). Supernatants that caused 100% cytotoxicity were considered positive.

Enterotoxin activity was measured as previously described using the suckling mouse assay and the rabbit ileal loop model (21,23). Cholera toxin cross-reactive factor (CTCRF) was measured in a ganglioside ELISA, using purified cholera toxin to produce a standard curve (22).

Enteroinvasion Assays

All A. hydrophila strains were assayed in the Sereny test to determine invasive potential (24). Overnight broth cultures (0.01 ml) were inoculated into the eye of adult Hartley strain guinea pigs. The animals were then examined daily for keratoconjunctivitis for 6 days. A second method was also used to screen these strains for invasive capabilities. Overnight broth cultures (1 ml) were injected into ligated rabbit ileal loops. The animals were sacrificed after 18 hours and the ileal loops were examined for evidence of invasion and fluid secretion. In both assays, a virulent Shigella sonnei was used as a positive control.

Hemagglutinaton Assays

Mannose-sensitive and mannose-resistant hemagglutination patterns were determined using the method of Evans et al. (25). Human type A, bovine, chicken, monkey and guinea pig erythrocytes were tested in both the presence and absence of mannose (Table 3).

Selection of Enteroadherent E. coli for Testing in Volunteers

Two strains of EAEC were selected for further study in volunteers, JM 189 and JM 221. The only recognized E. coli characteristic possessed by these strains was mannose-resistant adherence to HEp-2 tissue culture cells. These strains did not produce heat-labile or heat-stable enterotoxins; were Sereny test negative; and did not belong to recognized enteropathogenic somatic serogroups. Both JM 189 and JM 221 were isolated as the same pathogen from 2 U.S. travelers to Mexico. Each patient had a fourfold rise in serum antibody to the somatic antigen of the strain isolated from that patient. In the adherence assay, JM 189 adhered in a diffuse pattern and JM 221 exhibited localized adherence to the HEP-2 cells.

Volunteer Challenge Studies

Volunteers were identified through advertisement in local university newspapers and the 2 city-wide daily newspapers. The advertisements instructed potential volunteers to call a phone number to learn more about the study from

Table 3. Toxins Produced and Hemagglutination Patterns of *A. hydropnila* Strains

	Toxin Production					Hemagglutination Pattern Red Blood Cells Tested				
Strain	Hemolysin	Cytotoxin	Suckling Mouse	Rabbit ileal loop	CTCRF	Human	Bovine	Chicken	Monkey	Guinea Pig
6Y	+	+	+	+	+	-	-	-	-	-
B158	+	+	+	+	+	-	-	-	-	MS*
3647	+	+	+	+	+	-	-	-	-	-
SSU	+	+	-	-	+	-	-	-	-	-
3284	+	+	-	-	+	-	-	-	-	-

*Mannose-Sensitive nemagglutination only.

a recruiter. Interested persons were then asked to come to Methodist Hospital to hear details about the study a second time and to be scheduled for medical screening. During the screening process, a medical history form was completed and reviewed by a physician and a chest X-ray, EKG, and blood chemistry profile were performed. Consenting healthy adults were admitted and confined to the Methodist General Clinical Research Center. A prechallenge admission stool sample was cultured for enteropathogens as previously described (21).

Volunteers abstained from eating and drinking for the 90 minutes prior to and following oral challenge with test organisms. In a double blind study, groups of 3 or 4 volunteers were given 2 grams of $NaHCO_3$ in 150 mL sterile distilled water as follows: 120 mL of the bicarbonate solution was swallowed and 5 minutes later, the remaining 30 mL of bicarbonate containing the test organism at predetermined levels was digested. The subjects were carefully monitored for signs of diarrhea defined as 3 or more unformed stools within 24 hours, or 2 or more unformed stools in 24 hours accompanied by a sign or symptom of enteric infection. All stools passed were collected and examined for A. hydrophila or EAEC.

Results

Aeromonas Studies

Biochemical Characterization and Antimicrobial Susceptibility

All 5 strains were confirmed as A. hydrophila biochemically. The occurrence of several biochemical properties was noted, as potential indicators of virulence (Table 2). All of the strains gave a positive Vogues-Praskauer reaction; only 2 strains produced lysine decarboxylase; and hydrolyzed esculin and produced DNase. The strains were susceptible to the antimicrobial agents commonly used to treat acute bacterial diarrhea.

Toxin Production

All strains were hemolytic for sheep and rabbit erythrocytes and cytotoxic for YAC. Three strains (6Y, B158, and 3647) produced fluid accumulation in the suckling mouse and rabbit ileal loop assays. All 5 test strains produced a cholera-like toxin cross reactive factor that was detectable in the ganglioside ELISA.

Hemagglutination Assays

All 5 A. hydrophila strains failed to demonstrate mannose-resistant hemagglutination with any of the erythrocytes from the 5 animal species. Strain B158 showed mannose-sensitive agglutination for guinea pig erythrocytes.

Invasiveness

None of the A. hydrophila strains were able to produce keratoconjunctivitis in guinea pigs. All 5 strains did however produce fluid secretion in the rabbit ileal loops along with a purulent hemorrhagic discharge.

Table 2. Biochemical Characterization and Antimicrobial Susceptibility of A. hydropnila Strains

Strain	Biochemical Reactions				Antibiogram*						
	Vogues Praskauer Reaction	Lysine Decarb. Production	Esculin Hydrolysis	DNase Production	Am	Te	SXT	GM	Fx	DX	G
6Y	+	-	+	+	S	S	S	S	S	S	S
B158	+	-	+	+	R	S	S	S	S	S	S
3647	+	-	+	+	R	S	S	S	S	S	S
SSU	+	+	+	+	S	S	S	S	S	S	S
3284	+	+	+	+	R	S	S	S	S	S	S

*Am - Ampicillin, Te - Tetracycline, SXT - Trimethoprim/sulfamethoxazole, GM - Gentamicin, Fx - Furazolidone, DX - doxycycline, G - Sulfisoxazole.

Volunteer Challenge Studies

Using the 5 Aeromonas strains, we failed to demonstrate the development of diarrheal illness in 55 of 57 volunteers even with the doses of 10^{10} colony forming units (CFU) for 3 of the strains (Table 4). Three strains (B158, SSU and 3284) were not recovered from the stools of the volunteers that ingested them. Strain 6Y was recovered from 11 of the 20 volunteers challenged. One of the volunteers did develop diarrnea (6 unformed stools over 12 hours associated with a period of nausea, vomiting, anorexia and malaise) 48 hours after ingesting 10^9 CFU. This indvidual was not treated and failed to develop a progressive enteric illness. A small bowel biopsy obtained shortly after passing the unformed stool was normal histologically. No illness occurred among the 4 individuals ingesting a higher dose of 6Y (10^{10}) casting doubt on the importance of the illness of the individual seen at the lower dose. Strain 3647, earlier identified in a diarrheal stool from a patient in Australia, was recovered from 3 of the 16 volunteers challenged. One volunteer receiving a dose of 10^7 passed 3 soft stools over an 18 hour period of. This individual had mild abdominal cramps, but did not excrete the test organism. Illness did not occur as the dose was increased and the organism was administered to additional volunteers (Table 5).

Enteroadherent Escherichia coli Studies

None of the 24 volunteers had HEp-2 adherent E. coli in their stools prior to challenge. Table percentage shows the data for the volunteer studies and Table 6 characterizes the illness in those patients who developed diarrhea. Strain 189, at a dose of 7×10^6 was associated with diarrhea in 1 of 4 volunteers. Three of 4 individuals experienced other enteric symptoms including 3 with abdominal pain and malaise which lasted 3 days. Another volunteer had a single watery stool on the fifth day. All 4 individuals shed 289 in their stools; 1 for 1 day and 3 for the length of the 5 day study. At dose of 1×10^{10}, 189 was not associated with diarrhea in any of the 4 volunteers, although one had a single watery stool on the third day of the study. None of these volunteers had any additional enteric symptoms and all shed 189 in their stools for the 4 day duration of the study.

Strains 221, at a dose of 7×10^8 was associated with diarrhea in 2 of 8 volunteers. The incubation period were 34 and 46 hours. One volunteer passed 3 unformed stools in 23 hours and the other passed 9 loose stools over a period of 30 hours. These individuals had no other enteric symptoms. Three other volunteers had single unformed stools between the third and fifth days of the trial. Only 1 of 8 had an additional enteric symptom (abdominal pain accompanied by a single unformed stool on the third day). All indidivuals cutlured shed strain 221; 2 for 3 days, 1 for 4 days, 1 for 5 days, and 3 for 6 days. One individual, having no bowel movements during the study, did not have stool cultures performed.

Table 4. Administered Dose and Excretion of *A. hydropnila* in Volunteers

Strain	Challenge Dose	Number Volunteers	Number Shedding Test Strain	Diarrhea+
6Y	$2x10^4$	4	1	0
	$1x10^6$	4	1	0
	$7x10^7$	4	4	0
	$3x10^9$	4	3	1
	$4x10^{10}$	4	2	0
B158	$6x10^4$	4	0	0
	$2x10^7$	4	0	0
3647	$1x10^7$	4	0	1
	$4x10^7$	4	0	0
	$2x10^9$	4	2	0
	$3x10^{10}$	4	1	0
SSU	$4x10^8$	4	0	0
	$5x10^8$	3	0	0
3284	$3x10^8$	3	0	0
	$1x10^{10}$	3	0	0

+ - 3 unformed stools/24 hrs or 2 unformed stools/24 hrs with systemic or enteric symptoms.

Table 5. Enteroadherent Escherichia coli Volunteer Studies

Strain Number	Dose	Number with Number with Diarrhea[1]	Number Enteric Symptoms[2]	Shedding[3] (range in days)
189 (4)	7×10^8	1/4	3/4	4/4 (1-5)
189 (4)	1×10^{10}	0/4	0/4	4/4 (4)
221 (8)	7×10^8	2/8	1/8	7/7* (3-6)
221 (8)	1×10^{10}	3/8	5/8	8/8 (1-5)

1 diarrhea = 3 unformed stools/24 hr or 2 unformed stools/24 with an enteric symptom.

2 enteric symptoms = fever, malaise, vomiting or abdominal pain.

3 no volunteer shed EAEC prior to ingestion of the organisms.

* one volunteer did not furnish specimen.

Table 6. Characterization of Illness in Volunteers with Diarrnea due to Enteroadherent Escherichia coli

Test Organism	Dose	Incubation Period	No. Unformed Stools (Duration)	Enteric Symptoms (Duration)	Total Weight of Unformed Stools
189	7×10^8	52 hours	2 (12 hours)	chills, abdominal pain, malaise (3 days)	402.9g
221	7×10^8	46 hours	3 (23 hours)	None	687.5g
221	7×10^8	34 hours	8 (30 hours)	None	637.6g
221	1×10^{10}	41 hours	2 (7 hours)	abdominal pain (3 days)	240.8g
221	1×10^{10}	36 hours	5 (42 hours)	fever (101°F), nausea, vomiting, chills, abdominal pain, malaise (2 days)	536.0g
221	1×10^{10}	20 hours	4 (18 hours)	chills, nausea, abdominal pain, malaise (3 days)	223.3g*
Average		38 hours	4 (22 hours)	2.75 days	501.0g

* Two specimens were not collected.

At 1 x 10^{10}, 221 strain was associated with diarrhea in 3 of 8 volunteers. Incubation periods were 20, 36 and 41 hours. One passed 5 unformed stools during a 42 hour period. Another passed 4 unformed stools over 18 hours and the other experienced 2 loose stools over 22 hours. Four other individuals had unformed stools between the second and fifth day of the study. Five of the 8 volunteers at this nigher does experienced other enteric symptoms including 5 with abdominal pain, 3 with malaise, 2 with vomiting, and 1 with fever (101°C). One subject shed the test strain for 1 day and the remaining subjects shed the strain the entire length of the 5 day study.

Discussion and Conclusions

Aeromonas

A. hydrophila, an organism found commonly in the environment, has been statistically associated with diarrheal disease in man in a limited number of parts of the world. Its pathogenicity for selected patients is certain, particularly in skin infections and septicemia. We tested 5 representative strains of A. hydrophila based on their possession of well characterized virulence properties as well as their association with diarrheal disease. Diarrheal illness failed to occur in 57 volunteers fed varying doses of the characterized strains. The strains did not efficiently colonize the gut of the volunteers as demonstrated by the resultant fecal shedding patterns.

One of the two following conclusions appear to explain the results of the present study:

1. Virulence properties of A. hydrophila as we now understand them - biochemical characteristics and production of hemolysin, cytotoxin, and enterotoxins are insufficient to explain virulence for man.

2. Widespread immunity to A. hydrophila exists among adults from Houston, Texas.

We feel that the latter is not a reasonable explanation for failure to produce illness in view of the rarity of isolating Aeromonas from diarrheal stools of infants and children from Houston studied by our group over the past 10 years (26,27). Perhaps we are at the point with Aeromonas, in terms of understanding its virulence characteristics, where we found ourselves in the early 1970's for Escherichia coli. It is possible that the strains of Aeromonas we tested lack the necessary fimbriae to initiate colonization, the first step in pathogensis. Even though the strains possessed hemagglutinins, which may be associated with fimbriae, these fimbriae may not be intestinal epithelial cells adhesins for Aeromonas. Previously recommended biochemical testing (6) was not useful in differentiating strains with virulence for man. Additional virulence properties of Aeromonas strains need to be sought and identified before future volunteer studies are likely to be rewarding. Also, we know that enteric bacteria normally pathogenic for only infants (i.e., EPEC) can produce diarrhea in adults when given in the doses employed here (28).

Enteroadherent E. coli

Our studies have indicated that Escherichia coli which are identified only by their HEp-2 cell adherence property are as commonly associated with acute

diarrhea in young adults traveling to Mexico as are strains of shigella. That these strains actually caused the illness in this setting is suggested by a variety of findings: 1) the agents often were the sole agent identified in diarrheal stools; 2) they were isolated in a lower frequency from asymptomatic individuals when compared to illness cases; 3) humoral antibody development to somatic antigens of the organism commonly occurred during infection; and 4) a limited number of volunteers exposed in Houston to 10^8-10^{10} cells experienced clinical illness resembling the disease originally studied in Mexico.

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